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Article

Characterization of Anaerobic Degradability and Kinetics of Harvested Submerged Aquatic Weeds Used for Nutrient Phytoremediation

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Abstract: In this study, eight different submerged aquatic species were screened by batch biochemical methane potential and anaerobic degradability tests to identify a promising/suitable feedstock. Kinetics of the best-screened substrate were studied in a mesophilic semi-continuous experiment. The aquatic species *Myriophyllum aquaticum*, *Egeria densa* and *Potamogeton perfoliatus* showed relatively higher methane yields of over 400 NmL/g-VS (volatile solids). Semi-continuous operation was carried out by feeding *E. densa* for over 400 days. The achieved results were 33%–53% chemical oxygen demand (COD) reduction and methane yield of 126–231 NmL/g-VS with a short hydraulic retention time (HRT). Additionally, the NH_4^+ and PO_4^{3-} releases from the biomass to water were found to be low (18%–27% and 2.5%–3.9%) throughout the experiment. Hydrolysis was the limiting step in the digestion of *E. densa*, regardless of changes in HRT (15–45 days). The acid-phase model indicated that the hydrolysis rate constant (k_h) of *E. densa* was 0.058 one/day, which was one third lower the k_h value of food waste, but quite similar to cow manure.

Keywords: aquatic weeds; anaerobic digestion; limiting step; hydrolysis

1. Introduction

Nutrient phytoremediation using aquatic weeds has been considered a feasible method for secondary wastewater treatment or *in situ* water purification. Water pollution and eutrophication by excess nutrients owing to rapid industrialization and civilization are becoming big issues, especially in those countries where no well-systematized wastewater treatment plants are available to properly purify domestic wastewater. Thus, development of simple and low cost wastewater treatment technologies have attracted the interest of researchers and engineers. Aquatic weeds are capable of directly uptaking inorganic nutrients and also allow attachment of microorganisms and animals responsible for nutrient removal, so the weeds are effective in low-cost treatment of pollutants [1,2]. The nutrient uptake efficiency of this process depends on the weeds' ability to accumulate the nutrients as biomass. Therefore, repeated harvesting of the excessive biomass is necessary to maintain the high nutrient uptake efficiency. However, management or utilization of the harvested plant biomass is a primary issue of concern. When the plants decay, nutrients are released again to water body, and that has a negative impact on water quality [3]. Furthermore, excessive growth of aquatic weeds is conversely able to cause various problems such as interfering with ship navigation, releasing unwanted odors, blocking daylight to the organisms and deoxygenation of water leading to the death of fish and other aquatic life forms [4–6]. Harvesting and disposal of the excessive weeds are costly issues, for example removing 1 ton (wet weight) of weeds requires around 300 USD (US dollars), and 1200 tons of excessive weeds are removed every year from Lake Biwa, which is the biggest lake in Japan. Nowadays, cost-effective disposal and bio-resource utilization are required to build a sustainable treatment system.

Anaerobic digestion has been globally studied and considered as a cost-effective way of waste disposal, and therefore this technology has been widely installed, even in low-income rural areas [7]. Harvested aquatic weeds can be digested and used to successfully produce methane as a renewable energy source [4,5,8–10]. Aquatic plants generally contain a larger amount of biodegradable protein than other plants, and also have the possibility of allowing microorganisms to degrade substrate relatively rapid in anaerobic digestion. A lot of weed species have been utilized by researchers for anaerobic digestion, and it was found that the organic matter composition varied greatly depending on the weed species, which resulted in a wide range of methane yields among the different weed species (38–361 mL/g-volatile solids (VS) added) [9,10]. Like other plant species, aquatic weeds contain cellulose, hemicellulose and lignin, which are characterized by slow degradation rates in anaerobic digestion. Lignin is known to be hardly degraded during practical reaction times. Benner *et al.* [11] reported that only 1.5%–16.9% of lignin was degraded during over 200 days of anaerobic digestion. Thus, lignocellulosic material-rich weeds seem to limit biological methane production, so the choice of an appropriate species is important for effective methane production.

Until now, most researchers have concentrated on water hyacinth (*Eichhornia crassipes*) [8,12,13], and there is very limited available information about the anaerobic digestibility of other kinds of aquatic weeds [9,10]. Furthermore, most studies have focused on batch reactors, but in most practical cases, an anaerobic digester is operated at a semi-continuous or continuous mode. To the best of authors' knowledge, only two research groups have investigated the semi-continuous anaerobic digestion of water hyacinth as a floating aquatic macrophyte [8,14]. Additionally, the digestibility of submerged aquatic macrophyte species under semi-continuous operation remains unclear, as results from studies with other conventional batch methods suggested that there was a gap in methane yield during batch and semi-continuous experiments. Generally, the methane obtained from the semi-continuous experiment was rather lower than seen in the batch experiment [8]. Other aquatic weed species need to be investigated under semi-continuous operation conditions to expand and understanding their realistic digestibility. In another aspect, semi-continuous experiments are useful for discussing the kinetics of individual reaction steps in anaerobic digestion—hydrolysis, acidogenesis and methanogenesis—by considering the mass balance between influent and effluent streams. As discussed above, lignocellulosic materials have biodegradation difficulties, and in most cases hydrolysis becomes the limiting step [15,16]. Since the slow hydrolysis limits the digestion efficiency of plant materials, submerged aquatic weeds are expected to allow easy hydrolysis and rapid methane production due to their higher protein content than other plant species [10]. The reaction step that truly governs the entire reaction rate in anaerobic digestion of submerged aquatic weeds is not yet well explained. Therefore, the limiting step needs to be determined and interpreted in terms of degradation kinetics in semi-continuous experiments. Thus, the objective of this study is firstly to screen promising feedstocks for anaerobic digestion among eight different submerged aquatic weed species, and subsequently investigating the anaerobic degradability and degradation kinetics of the weeds in a long-term semi-continuous anaerobic digestion.

2. Results and Discussion

2.1. Screening of Submerged Aquatic Weed Species Used for Anaerobic Digestion Based on Batch Biochemical Methane Potential Tests

Batch digestion experiments were run in triplicate for 60 days using eight different submerged aquatic weed species (*Hydrilla verticillata*; *Potamogeton inbaensis*; *Potamogeton dentatus*; *Potamogeton malaianus*; *Ceratophyllum demersum*; *Potamogeton perfoliatus*; *Myriophyllum aquaticum*; *Egeria densa*) to screen a suitable feedstock for anaerobic digestion. *H. verticillata*, *P. inbaensis*, *P. dentatus*, *P. malaianus*, *C. demersum*, *P. perfoliatus* are all native species and common macrophytes observed at lakes and waterways in Japan. On the one hand, *M. aquaticum* and *E. densa* are popular invasive alien species and they often cause excessive growth problems in lakes of Japan. In addition, it has been previously demonstrated that these eight plant species are useful for nutrient remediation. The batch experiment of *E. crassipes* was additionally conducted as a contrastive feedstock, which has been well investigated. The aquatic weeds mainly consisted of protein and cellulose (Table 1). There was a slight variation in cellulose concentrations among the plant species, and the concentrations ranged from 178 to 212 mg/g-total solids (TS). A significant level of hemi- cellulose was contained in *H. verticillata* (66.0 mg/g-TS) and *C. demersum* (82.0 mg/g-TS).

Table 1. Characteristics and composition of the submerged macrophytes.

Species	Cellulose (mg/g-TS)	Hemicellulose (mg/g-TS)	Lignin (mg/g-TS)	Protein (mg/g-TS)	Lipid (mg/g-TS)	Carbon/nitrogen ratio
P. malaianus	212	Not detected	116	350 ± 39	34.2	12.5 ± 0.3
P. perfoliatus	200	Not detected	165	298 ± 32	26.1	8.5± 0.2
C. demersum	185	82.0	186	315 ± 121	31.8	10.4 ± 0.2
P. dentatus	195	Not detected	155	266 ± 39	41.0	8.7 ± 0.1
H. verticillata	178	66.0	129	261 ± 86	15.1	9.6 ± 0.4
E. densa	202	Not detected	50	294 ± 24	29.1	10.2 ± 0.2
P. inbaensis	210	Not detected	83	280 ± 32	49.4	12.9 ± 0.4
M. aquaticum	200	Not detected	59	286 ± 33	53.8	9.8 ± 0.3

Lignin content of all the aquatic weeds was relatively low (50–186 mg/g-TS). The protein concentrations varied from 261 to 350 mg/g-TS among the different species and *P. malaianus* and *C. demersum* exhibited the higher concentration levels. Lipid concentrations were low (15 to 54 mg/g-TS). Figure 1 shows cumulative methane production performance for 60 days per g-VS added and theoretical maximum methane production, which was calculated from theoretical methane value of 350 mL-CH_4/g-chemical oxygen demand (COD) of substrate. The results showed more or less similar cumulative methane production patterns between the nine species. Almost half of the total methane production was obtained within the first 4 days, and the gas production reached saturation at day 36. As a result, the volumes of methane varied from 275 to 418 NmL/g-VS. *E. densa*, *M. aquaticum* and *P. perfoliatus* had relatively higher methane yields over 400 NmL/g-VS while the yield of *E. crassipes* was the lowest (275 NmL/g-VS). Other weeds yielded in the range of 334–355 NmL/g-VS. Compared to the theoretical maximum production, the methane production from the weeds in the experiment reached approximately 70%–90% of the theoretical values. It is clear from these results that all the eight submerged plant species had higher methane yields than *E. crassipes* which is used as a conventional aquatic weed fed to digesters. This is likely due to the higher protein content of the submerged weeds, which are thus relatively easy to degrade. Although *E. crappipes* is known for its low lignin content, which was below 15 wt% in previous studies [17,18], it mostly consists of cellulose and hemicellulose. The protein content of *E. crappipes* reported elsewhere was rather lower (below 13 wt%) than those of the weed used in this study [17,18].

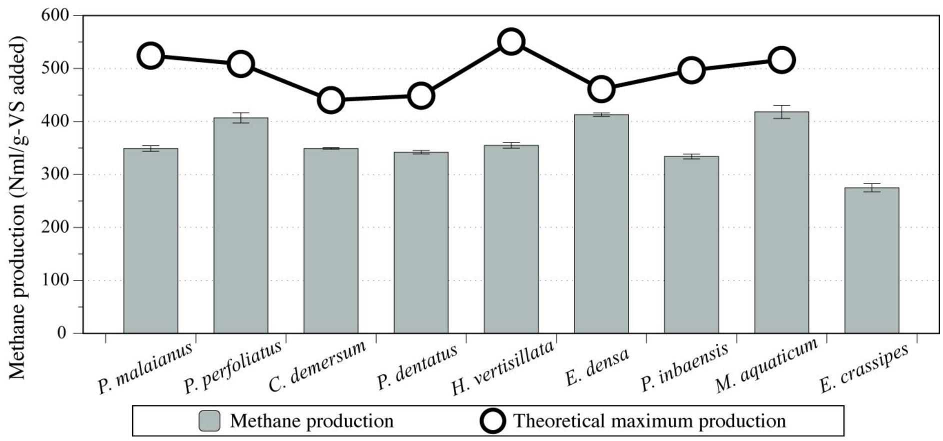

Figure 1. Cumulative methane production of different aquatic plant species.

The methane yields obtained in this study were in accordance with earlier studies investigating various floating and submerged aquatic weeds. For example, the yield of *E. crassipes* was within the range of 150–300 mL/g-VS [4,8,14,19]. Koyama *et al.* [10] examined the methane production potentials of several submerged aquatic weed species, including *C. demersum*, *E. nuttallii*, *E. densa*, *P. maakianus* and *P. malaianus* under mesophilic condition (37 °C), and reported productions of 161–361 mL/g-VS during a period of 14 days. In that research, among the five species studied, *E. nuttallii* had the highest methane yield (361 mL/g-VS) and *E. densa* had the second highest yield (287 mL/g-VS). This result is quite similar to the results achieved in this study, as *E. densa* showed a higher methane yield than *C. demersum* and *P. malaianus*, and moreover, *E. densa* produced about 300 NmL/g-VS of methane within 14 days. It is well known that *E. densa* has a high growth rate and inhibits a wide range of aquatic systems [20,21]. For these reasons, *E. densa* is thought to be one of the promising substrates for anaerobic digestion in respect of not only methane yield, but also biomass productivity.

Generally, methane yield in anaerobic digestion is positively correlated to lipid/protein contents and negatively to lignocellulosic material content. In a previous study, it was found that lipid/protein rich feedstocks showed higher methane yields than cellulose-rich ones [22]. Koyama *et al.* [10] observed a negative correlation between methane yield and lignin content among several submerged aquatic weeds. However, it was difficult to find a statistically significant relevance between any organic compounds and methane yield in anaerobic digestion of this study. Nevertheless, it should be noted that *E. densa* and *M. aquaticum*, which showed higher methane yields, were characterized by their low lignin content of below 60 mg/g-TS. However in the case of the other samples, there was no significant correlation between methane yield and lignin content.

2.2. Performance of Mesophilic Anaerobic Digestion Treating E. Densa in Semi-Continuous Operation

2.2.1. Characteristics of Feedstock (*E. Densa*)

E. densa, which showed higher methane production in the batch experiment, was selected as the feedstock for semi-continuous operation because it was an invasive and one of the most predominant aquatic weed species in the major lakes and rivers of Japan. Feedstock was prepared by diluting dry *E. densa* with 10-fold the amount of tap water. The feedstock possessed 89–94 g/L-TS, 76–79 g/L-VS, 88–94 g/L COD. The carbon/nitrogen (C/N) ratio was 10 on average. Total nitrogen (T-N) and total phosphorus (T-P) concentrations were varied within the range of 2500–3800 mg/L, 260–510 mg/L throughout the experiment, respectively. The Fe, Ni, Co concentrations were 4.0 ± 1.2, 0.0053 ± 0.0025 and 0.0014 ± 0.0006 mg/g-TS (nearly equal to minimum detection limit of the equipment), respectively.

2.2.2. Time Course of Gas Production and Digestibility during the Experiment

A semi-continuous digestion experiment of *E. densa* was performed over 400 days by gradually shortening the hydraulic retention time (HRT) from 45 to 15 days in a continuously mixed reactor. Average organic loading rates (OLR) corresponding to each HRT were 2.01 ± 0.05 (45 days), 3.05 ± 0.08 (30 days), 4.56 ± 0.13 (20 days) and 6.24 ± 0.07 (15 days) kg-COD/m^3/day, respectively. Figure 2 indicates the time course of gas production rate and gas composition throughout the experiment. The gas production rate was slightly increased according to decrease in HRT from 45 to

30 days. However, it maintained a similar level during HRTs of 20 and 15 days. Methane and CO_2 concentrations showed a variation from 40% to 60% throughout the experiment. The pH was maintained within a range of 7.4–7.7 during the whole operation without any chemical pH control. The volatile fatty acids (VFA) was generally below 300 mg/L as acetate, but increased a little over 1000 mg/L (three times higher) during HRTs of 20 and 15 days.

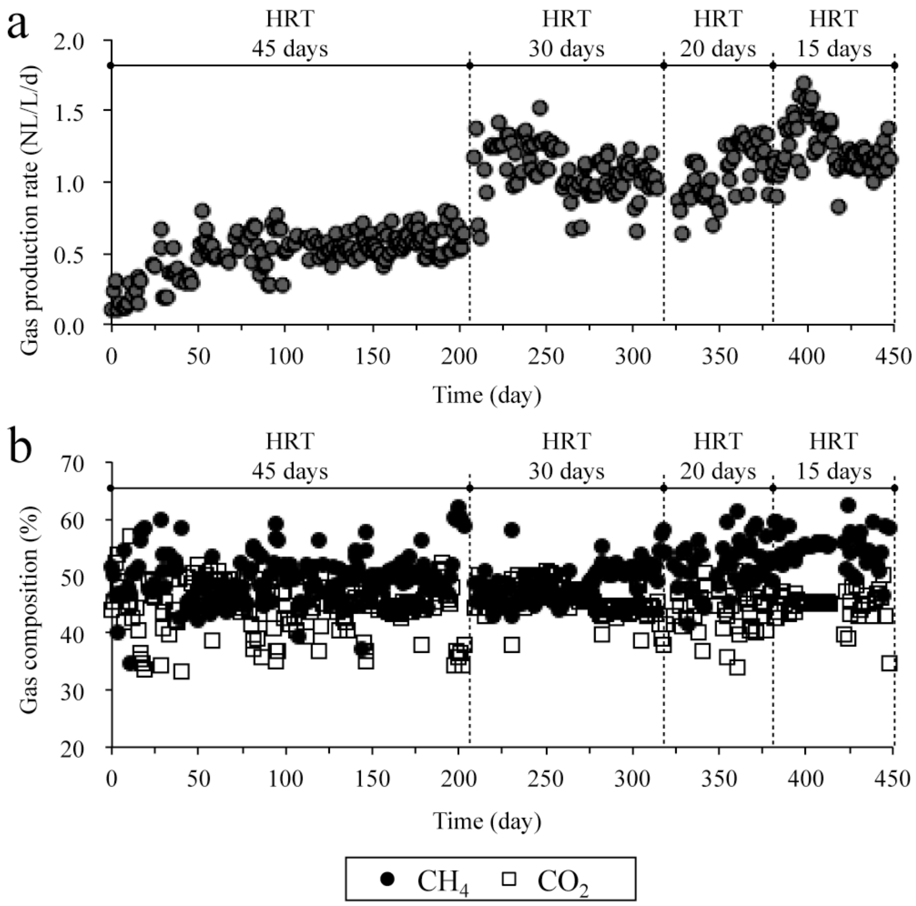

Figure 2. (**a**) Time courses of the biogas production rate; and (**b**) CH_4 and CO_2 concentrations in the biogas throughout the semi-continuous experiment. HRT: hydraulic retention time.

2.2.3. Characteristics of Organic Compounds Degradation

Table 2 summarizes the characteristics of organic compound degradation during the anaerobic digestion of *E. densa*. The evaluation was carried out during the last 10 days of each HRT-operation. The average methane production rate was increased when the HRT was shortened from 45 to 20 days, and decreased when the HRT was 15 days. The methane yield decreased from 231 (45 days HRT) to 126 NmL/g-VS (15 days HRT). The COD reduction decreased gradually from 52.7% to 33.1% with shortening HRT. The protein degradation also showed a similar variation, and maintained higher levels than that of COD during the experiment. The NH_4^+ and PO_4^{3-} release were in the ranges of 18%–27%, 2.5%–3.8%, respectively. There has been a smaller variation in methane yield, COD and protein degradations, and NH_4^+ and PO_4^{3-} release, respectively. Firstly, it should be noted that *E. densa* was characterized by very low solubilization of nitrogen and phosphorus as NH_4^+ and PO_4^{3-} released from biomass to water. For example, the NH_4^+ release from food waste was 68%–77% in our previous study [23] (data not shown). One of the important roles of submerged aquatic weeds in a phytoremediation system is uptaking nitrogen and phosphorus from the water bodies. As such, the less NH_4^+ and PO_4^{3-} releases result in reduced re-outflow of fixed nutrients of the weeds via anaerobic digestion, which is considered preferable.

Table 2. Effect of HRT on the degradation characteristics in the semi-continuous experiment.

Parameter	HRT (days)			
	45	30	20	15
Methane production rate (NL/L/d)	0.38 ± 0.06	0.53 ± 0.04	0.67 ± 0.08	0.64 ± 0.04
Methane yield (NmL/g-VS added)	231 ± 24	201 ± 10	171 ± 21	126 ± 7
COD reduction (%)	52.7 ± 8.2	44.6 ± 2.4	38.2 ± 0.9	33.1 ± 1.8
Protein reduction (%)	62.1 ± 6.7	53.9 ± 3.3	47.8 ± 3.7	38.7 ± 13.2
NH_4^+ release (%)	23.4 ± 3.3	22.0 ± 5.7	26.8 ± 2.7	18.0 ± 3.2
PO_4^{3-} release (%)	2.5 ± 1.5	2.5 ± 0.5	3.9 ± 0.9	3.8 ± 1.7

However, the energy recovery was less than expected. The methane yield (126–231 NmL/g-VS) obtained from the semi-continuous experiment was lower than in the batch experiment (413 NmL/g-VS). In addition, it is suggested lower methanogenic rate or yield of the semi-continuous reactor that it took 30 days of retention time to produce 201 NmL/g-VS methane in the semi-continuous operation while 200 NmL/g-VS methane was produced within the first 4 days of the batch experiment. Similar results have been obtained in other studies using different aquatic weeds [8,14]. The biogas yield of the semi-continuous experiment fed with water hyacinth was about half of the methane potential (340 mL-CH_4/g-VS) observed in the batch experiment [8]. Srivastava [14] reported similar biogas yield of around 300 mL-biogas/g-VS in a semi-continuous experiment feeding water hyacinth. Chanakya *et al.* [12] also reported low methane yields (292–348 mL-biogas/g-VS with 50% CH_4) in the semi-continuous experiment. From these outcomes, it seems to be a common issue that aquatic weeds virtually produce rather less methane during practical retention times (<30 days) of anaerobic digestion than the saturation level which those weeds can potentially produce. By contrast, the batch experiment enabled the faster methane production and reached the saturation in a shorter period even under a high feed to inoculum (F/I) ratio (0.5). These facts suggest that there was a significant difference in methanogenic activity or cell density between the batch reactors and semi-continuous reactors, and thus, it took longer time to degrade enough *E. densa* in the semi-continuous operation. The possible reason for the slow methane production of the semi-continuous experiment is likely due to lower activity of microbial consortia. The seed sludge used for batch experiment was taken from the reactor fed with food waste and supplementary micronutrients (Fe, Co, Ni). Although the batch experiment was carried out under nutrient-rich condition (C/N ratio, macro or micronutrients content were suitable), semi-continuous operation was probably under nutrient-limited conditions. In this research, firstly, less available macronutrients (C, N, P) due to low organic degradation efficiency of *E. densa* likely resulted in reduced microbial growth in the reactor. As Table 2 shows, P is especially limited. The PO_4^{3-} concentration was low as in most cases 1–5 mg-phosphorus (P)/L and even NH_4^+ concentration was usually below 1000 mg-N/L, while those in the food waste-digestion were about 1500–4000 mg-N/L and 60–200 mg-P/L in the previous study [24]. Furthermore, low concentration of micronutrients such as Fe, Co could contribute to limit activity of microbial consortia. Martinez and Martinez and Church [25] demonstrated that Fe and Co added to the media significantly stimulated cellulose digestion. Qiang *et al.* [26] calculated the required Fe/COD, Co/COD, Ni/COD ratio in the solid waste substrate for anaerobic digestion, and suggested that Fe/COD, Co/COD, Ni/COD were required in the quantities of 200, 6.0 and 5.7 mg/kg, respectively. In this study, the Fe/COD, Co/COD, Ni/COD ratios of *E. densa* were 3900, 1.4, 5.1 mg/kg, which indicated that the Co/COD was significantly lower than the required. The Co shortage was likely to negatively affect anaerobic cellulose degradation. Moreover, there was the possibility that biologically available C/N ratio was too high due to the very low dissolved nitrogen release (317–934 mg/L NH_4^+-N and 331–898 mg/L dissolved T-N in the reactor) of *E. densa* during anaerobic degradation. The C/N ratio of *E. densa* was lower than conventional digestion feedstocks, but the low concentration of released NH_4^+ as available nitrogen source might result in reduced working C/N ratio. These factors might restrict microbial growth and activity, resulting in lower methane yield in the semi-continuous experiment. Addition of nutrient seems to be necessary as mentioned by O'Sullivan *et al.* [4], in the anaerobic digestion of water hyacinth, *Cambomba* and *Salvinia*. The authors concluded that the nutrient addition did not significantly contribute to batch digestion of aquatic weeds, however, the semi-continuous digestion would require additional nutrients for the reasons discussed above. Finally, another possibility is a reduced methanogenic activity due to toxic substances produced by the weeds. It is widely known that aquatic weeds, including *E. densa*, cause a growth inhibition of micro- and macro-organisms by allelopathic effects [21]. Bio-assays using various species of weeds have demonstrated that *E. densa* has an inhibitory effect on algal growth, and the intensity of inhibition varied between the algal species [27,28]. However, there has been no report investigating inhibitory effects of *E. densa* on methanogenic microbial consortia yet. Further studies are needed to understand inhibitory effect of aquatic weeds on anaerobic digestion.

2.2.4. Rate Limiting Step and Effect of HRT on the Reaction Steps

Figure 3a summarizes the reaction rates of three digestion steps in the semi-continuous experiment. All the reaction rates were increased during HRT ranging from 45 to 20 days. When the HRT became 15 days, the methanogenesis rate dropped from 1.95 to 1.82 g-COD/L/day while the hydrolysis and acidogenesis rates were increased. It is common in continuous operation of anaerobic digestion that methanogenic activity is decreased under too short HRT due to the low growth rate of methanogenic archaea, and in this case, the 15 days HRT seems to be too short for methanogenic archaea to retain enough microbial cells in the reactor. A comparison between the hydrolysis, acidogenesis and methanogenesis efficiencies clearly indicates that hydrolysis was the limiting step at any HRT length. Even when HRT was as long as 45 days, the gap between the three reaction rates did not become narrow. In anaerobic digestion, solid particles of *E. densa* were hydrolyzed first, and then, soluble contents produced were converted into volatile acids and hydrogen. Finally, methane and CO_2 were generated from acetic acid and hydrogen. When it comes to the details of this reactor, the acetic acid and propionic acid was the main VFA remained in the reactor, and there was no significant amount of hydrogen detected in the biogas. Although an accumulation of dissolved COD means acidogenesis from dissolved COD content to VFA was limited, the dissolved COD in the reactor remained within

1.0–4.3 g/L under all the HRT, and no significant variation was observed, which means successful acidogenesis ocurred throughout the experiment. Acetic acid was 100–200 mg/L on average, regardless of HRT, but propionic acid was increased about 100–1000 mg/L when HRT was 15 days. The accumulation of propionic acid is a common phenomenon that causes limited methanogenic activity. These data supports that hydrolysis was generally limited, resulting in no significant accumulation of the intermediates present, and methanogenesis rate was reduced when the HRT was 15 days.

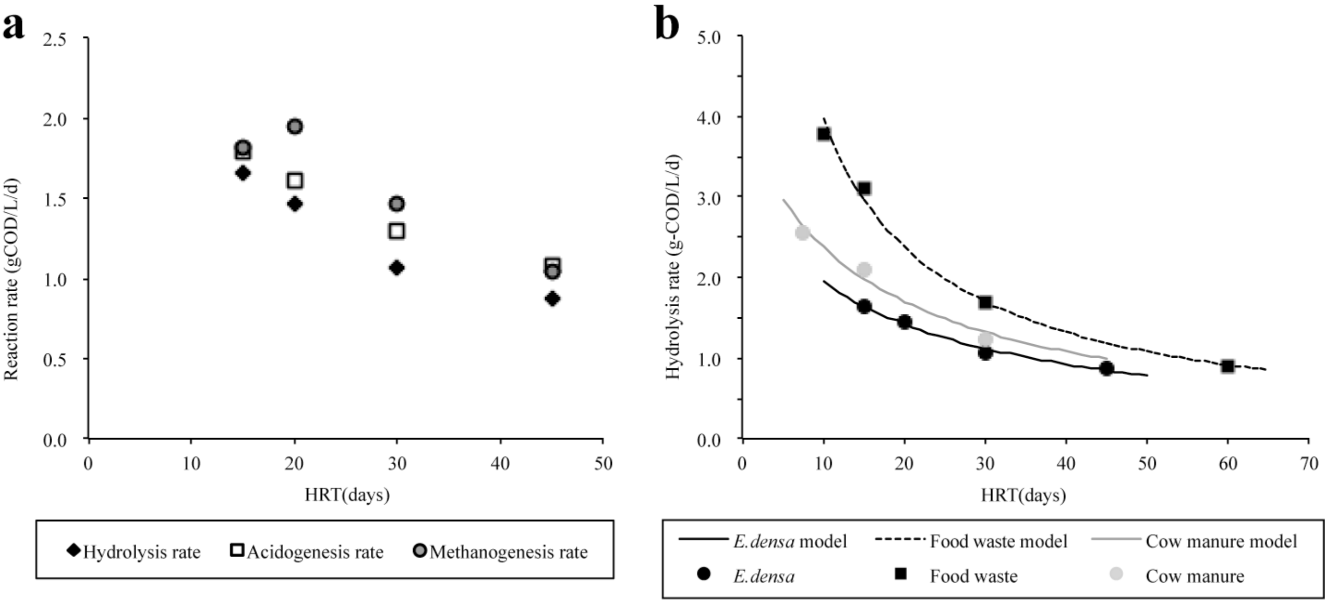

Figure 3. (**a**) The reaction rates of hydrolysis, acidogenesis and methanogenesis in the semi-continuous experiment; (**b**) The hydrolysis rate variations based on acid-phase model.

The hydrolysis rate constant (k_h) of the semi-continuous experiment was calculated using the acid-phase model and was compared with the k_h values obtained from our previous studies dealing with conventional digestion substrates [24,29]. All three substrates contained around 100 g/L TS and these were digested in a semi-continuous mode using mesophilic continuously mixed reactors. From the model, the k_h values were determined as 0.058 one/day (*E. densa*), 0.20 one/day (food waste) and 0.066 one/day (cow manure), and the biologically degradable fractions as particulate COD were 62.8% (*E. densa*), 89.8% (food waste) and 59.7% (cow manure), respectively. Figure 3b summarizes the hydrolysis rates of the three substrates and the calculated values. The k_h of *E. densa* was found to be lower than those of cow manure and below one third of that of food waste. This result indicates the hydrolysis rate of *E. densa* was lower than those of the other conventional feedstocks, but, attention should be paid to the fact that *E. densa* was comparable in reaction rate to cow manure as a conventional substrate without any special pretreatment. Meanwhile, as the biologically degradable fractions values suggested, an extended digestion would enable *E. densa* to achieve higher hydrolysis efficiency resulting in greater methane yield than that of cow manure. *E. densa* seems to consist of slow-biodegradable organic matter, and that is partially due to the high cellulose content. Generally, hydrolysis is widely considered as the rate-limiting step in anaerobic digestion of solid cellulose [15]. The hydrolysis kinetic studies on anaerobic digestion of cellulosic materials have provided very low k_h

values. The k_h values of office paper, cardboard, newsprint, hay, and straw were 0.036, 0.046, 0.057, 0.01 and 0.087 one/day, respectively [30–32]. *E. densa* had the similar but a little higher level of k_h than those of the other previously studied cellulosic feedstocks.

3. Materials and Methods

3.1. Biochemical Methane Potential Tests

A long-term outdoor experiment of wastewater purification processes using different kinds of aquatic plants was conducted, and the results have been partially reported elsewhere [33]. The processes have been fed with a mixture of domestic wastewater and water from Lake Kasumigaura. The inoculated plants were as follows: (1) *H. verticillata*; (2) *P. inbaensis*; (3) *P. dentatus*; (4) *P. malaianus*; (5) *C. demersum*; (6) *P. perfoliatus*; (7) *M. aquaticum*; (8) *E. densa*; and (9) *E. crassipes*. These plants were cultivated independently in parallel. The excess biomass harvested from the plants was used as feedstock for batch anaerobic digestion experiments. After draining the water off from the harvested plants, they were dried in the air for a day, and disintegrated by an electric blender to below 5 mm in length to allow reproducible results by dispersing the plant bodies. Subsequently, the feedstocks were diluted with tap water to make up the TS as approximately 10%.

Anaerobic digested sludge obtained from a mesophilic digester treating food waste was used as an inoculum after acclimating with the mixture of nine aquatic plants over three months at 35 °C. The F/I ratio based on feedstock VS and inoculum volatile suspended solids (VSS) was 0.5. The feed and inoculum were added to the closed glass vials with a working volume of approximately 60 mL, subsequently the vials were flushed by nitrogen gas and sealed with butyl rubber stoppers and aluminum caps to create a strictly anaerobic environment. The vials were incubated in shaking incubators at 35 °C with a shaking speed of 120 rpm. The gas production was measured using a glass syringe equipped with a needle. In this experiment, all batch tests were performed in triplicate, and vials containing only the inoculum were used as blanks. Besides, the biogas production from the blank was subtracted to correct for the biogas production from the inoculum.

3.2. Semi-Continuous Anaerobic Digestion Experiment of E. Densa

A continuously stirred reactor was used for the semi-continuous anaerobic digestion of *E. densa*. The reactor was made of PVC and had a working volume of 1 L. Sludge in the reactor was continuously mixed by an impeller at a mixing speed of 300 rpm. Hot water was circulated through the reactor's water jacket space to maintain a stable temperature of 35 °C. The feedstock *E. densa* was harvested every one or two months throughout the year from a water purification testing flume, which was 20 m in length and 5 m in width, semi-continuously fed with the mixture of domestic wastewater and lake water. After harvesting, the plants were drained and disintegrated by an electric blender. The pretreated *E. densa* were stored at 4 °C as dried state and diluted with tap water to make up the TS as approximately 10% when it was fed into the reactor. On the first day of the experiment, the anaerobic sludge obtained from a digester treating food waste was added to the reactor. Sludge withdrawal and substrate feeding were performed manually with a syringe every day at a given time.

3.3. Analytical Methods

Biogas production rate of the reactors was measured by a PMMA (poly(methyl methacrylate)) Milli Gas-counter (Ritter, Buckinghamshire, UK). The proportions of CH_4, CO_2 and N_2 in the biogas were determined using a gas chromatograph (GC-8A, Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector. The stainless steel column in the gas chromatograph was packed with Shicarbon ST (Shimadzu GLC). The COD concentration was determined using COD Digest Vials (HACH, Loveland, CO, USA) in accordance with the manufacturer's instructions. TS and VS were determined according to the U.S. Environmental Protection Agency (EPA) Standard Method [34]. Cellulose and hemicellulose concentrations were measured by the method of Van Soest and McQueen [35], and lignin was measured according to Effland [36]. The pH was determined as soon as sampling using a pH meter (TOA-DKK, Tokyo, Japan) equipped with a GST-5721C probe. The VFA concentration was determined using a gas chromatograph (GC14B, Shimadzu) equipped with a flame ionization detector and a StabiliwaxR-DA capillary column (Restek, Bellefonte, PA, USA). Nitrogen in the form of ammonium ion (NH_4^+–N) was analyzed using the automatic nutrient analyzer (Traacs 2000, Bran + Luebbe, Norderstedt, Germany). Samples for VFA and NH_4^+–N analysis were prepared by centrifuging sludge samples at 13000 rpm for 5 min and filtering them through 0.45 μm pore-size filters. The elemental composition of C and N was analyzed using an elemental analyzer (EA 1112, Thermo Fisher Scientific, Waltham, MA, USA). Metal concentrations of the weed were determined by inductively coupled plasma atomic emission spectrometer (61E-Trace, Thermo Jerrell Ash/Baird, Franklin, MA, USA) The C, N and metal measurements were performed via a fundamental measurement service of National Institute for Environmental Studies.

COD and protein reduction were calculated according to the way in the previous study [23]. NH_4^+ and PO_4^{3-} release from the weed were defined using the following equations:

$$NH_4^+ \text{ release}(\%) = 100 \times \frac{NH_4^+\text{-}N_{eff} - NH_4^+\text{-}N_{inf}}{T\text{-}N_{inf} - NH_4^+\text{-}N_{inf}} \quad (1)$$

$$PO_4^{3-} \text{ release}(\%) = 100 \times \frac{PO_4^{3-}\text{-}P_{eff} - PO_4^{3-}\text{-}P_{inf}}{T\text{-}P_{inf} - PO_4^{3-}\text{-}P_{inf}} \quad (2)$$

where $T\text{-}N_{inf}$ and $T\text{-}P_{inf}$ are the influent T-N and T-P, respectively.

Hydrolysis, acidogenesis and methanogenesis rate based on COD was defined according to the acid-phase model [33] by the following equations:

$$\text{Hydrolysis rate (g-COD/L/day)} = Q(COD_{parinf} - COD_{pareff})/V \quad (3)$$

$$\text{Acidogenesis rate (g-COD/L/day)} = Q(COD_{VFAeff} - COD_{VFAinf} + (COD_{inf} - COD_{eff}))/V \quad (4)$$

$$\text{Methanogenesis rate (g-COD/L/day)} = COD_{CH_4}/V \quad (5)$$

where Q is the flow rate of substrate (L/day) and V is the reactor volume (L). COD_{inf} and COD_{eff} are the influent and effluent COD, respectively. COD_{CH4} is the COD of methane produced per day. COD_{par} is the particulate COD (COD_{par} = COD − COD_{dis}), COD_{dis} is the dissolved COD, and COD_{VFA} is the COD of VFA. In the Equation (4), (COD_{inf} − COD_{eff}) indicates the COD, which was converted from VFA into methane. In these equations, bacterial growth was neglected although the original acid-phase model considered it. Hydrolysis of particulate COD in the semi-continuous experiment under steady-state conditions was considered according to a first-order reaction presented as follows [37,38]:

$$\frac{dF}{dt} = -\mathrm{k_h} F \quad (6)$$

$$Q\,(F_0 - F_{\mathrm{eff}}) - V\mathrm{k_h} F_{\mathrm{eff}} = \mathrm{HRT}(F_0 - F_{\mathrm{eff}}) - \mathrm{k_h} F_{\mathrm{eff}} = 0 \quad (7)$$

$$\mathrm{HRT} = F_0 \left(\frac{\mathrm{HRT}}{F_0 - F_{\mathrm{eff}}} \right) - \frac{1}{\mathrm{k_h}} \quad (8)$$

where F is the concentration of particulate organic matter, k_h is the hydrolysis rate coefficient and F_0 is initial concentration of degradable particulate organic matter. F_0 and k_h based on COD were obtained from a plot of HRT/($F_0 - F_{eff}$) and HRT using the slope and intercept. Although F_0 was unknown, $F_0 - F_{eff}$ can be calculated by the following equation:

$$F_0 - F_{\mathrm{eff}} = (\mathrm{COD_{infpar}} + \mathrm{COD_{inapar}}) - (\mathrm{COD_{effpar}} + \mathrm{COD_{inapar}}) = \mathrm{COD_{infpar}} - \mathrm{COD_{effpar}} \quad (9)$$

where COD_{inapar} is inert particulate COD. Although COD_{inapar} is unkown, ($F_0 - F_{eff}$) can be calculated using only COD_{infpar} and $COD_{effpart}$.

4. Conclusions

This study has demonstrated that *M. aquaticum*, *E. densa* and *P. perfoliatus* have relatively higher methane yields of over 400 NmL/g-VS. *M. aquaticum* and *E. densa* showed higher production due to their low lignin content characterized in the batch experiments. Semi-continuous operation over 400 days using *E. densa* resulted in 33%–53% COD reduction and 126–231 NmL/g-VS methane yield, besides, the performance increased when the HRT was shortened from 45 to 15 days. The low levels of NH_4^+ and PO_4^{3-} release were maintained throughout the experiment, indicating reduced re-outflow of nutrients via phytoremediation in anaerobic digestion. Hydrolysis was the limiting step in the digestion of *E. densa* and the gap between the three reaction rates was maintained in the range of 20–45 days HRT. The acid-phase model indicated that the k_h of *E. densa* was 0.058 one/day, which was lower than conventional feedstocks and a little higher than those of other cellulosic feedstocks.

Author Contributions

Takuro Kobayashi designed and carried out all the experiments and wrote the manuscript. Ya-Peng Wu performed the semi-continuous experiment. Zhi-Jiang Lu performed the batch experiment. Kai-Qin Xu designed the experiment and edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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